

A histopathological and biochemical evaluation of oxidative injury in the sciatic nerves of male rats exposed to a continuous 900-megahertz electromagnetic field throughout all periods of adolescence[☆]

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ABSTRACT

The effects on human health of the electromagnetic field (EMF) emitted by mobile phones, used by approximately 7 billion people worldwide, have become an important subject for scientific research. Studies have suggested that the EMF emitted by mobile phones can cause oxidative stress in different tissues and age groups. Young people in adolescence, a time period when risky behaviors and dependences increase, use mobile phones more than adults. The EMF emitted by mobile phones, which are generally carried in the pocket or in bags when not in use, will very probably affect the sciatic nerve. No previous study has investigated the effect of mobile phone use in adolescence on peripheral nerve. This study was planned accordingly. Twenty-four male Sprague Dawley rats aged 21 days were divided equally into control (CGr), Sham (SGr) and EMF (EMFGr) groups. No procedure was performed on CGr rats. EMFGr were exposed to the effect of a 900-megahertz (MHz) EMF for 1 h at the same time every day between postnatal days 21–59 (the entire adolescent period) inside a cage in the EMF apparatus. SGr rats were placed inside the cage for 1 h every day without being exposed to EMF. All rats were sacrificed at the end of the study period, and 1 cm sections of sciatic nerve were extracted. Malondialdehyde (MDA), glutathione, catalase (CAT) superoxide dismutase (SOD) values were investigated biochemically in half of the right sciatic nerve tissues. The other halves of the nerve tissues were subjected to routine histopathological tissue procedures, sectioned and stained with hematoxylin and eosin (H&E) and Masson's trichrome. Histopathological evaluation of slides stained with Masson's trichrome and H&E revealed a normal appearance in Schwann cells and axons in all groups. However, there was marked thickening in the epineurium of sciatic nerves from EMFGr rats. MDA, SOD and CAT levels were higher in EMFGr than in CGr and SGr at biochemical analyses. Apoptotic index (AI) analysis revealed a significant increase in the number of TUNEL (+) cells when EMFGr was compared with CGr and SGr. In conclusion, our study results suggest that continuous exposure to a 900-MHz EMF for 1 h throughout adolescence can cause oxidative injury and thickening in the epineurium in the sciatic nerve in male rats.

1. Introduction

Technological advances have led to a remarkable increase in the variety and numbers of devices using or sources of electromagnetic field (EMF), which raise profits for manufacturers and simplify consumers' lives (Mild et al., 2004). Mobile phones, which we constantly carry on our persons, are one such EMF-emitting device (Kerimoğlu et al., 2016a; Mugunthan et al., 2016). Verbal communication via mobile phones, a ubiquitous component of modern life, has become faster and easier thanks to technological add-ons being developed every day. Mobile phones, which are used for a variety of purposes, have become

ever more popular with users from all age groups due to their attractive features (Mugunthan et al., 2016). Multifunction smart phones in particular are one indispensable component of modern life. However, in addition to the benefits bestowed by these marvels of technology, there are also concerns over health risks due to the EMF effect they emit (Mild et al., 2004).

EMF can induce oxidative stress in tissues (Türedi et al., 2015; Kerimoğlu and Odacı, 2017; Bilgici et al., 2013). Oxidative stress is the cellular result of excessive reactive oxygen species (ROS) known as free radicals, and causes injury to vital structures and functions in the cell. Free radicals are molecules or ions with one or more unpaired electron

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in their external orbitals and are highly reactive. These are physiological products of cell metabolism. Low or moderate concentrations are beneficial to the organism due to roles in protection against micro-organisms and cellular signaling mechanisms (Valko et al., 2007). However, the excessive radical production and the oxidative stress originated from it are known to cause various degenerative disorders. These oxygen radicals may lead to damage in DNA, proteins and lipid membranes (Moller et al., 1996; Anderson, 1996; Valko et al., 2007).

The effect of EMF on the body depends on the frequency and strength of the waves. The speed at which EMF changes direction determines the frequency, which is expressed as Hertz (Hz). One of the most widely used cellular communication systems is the Global System for Mobile Communications-900 (GSM-900), which operates at a frequency band of 880–960 MHz. A frequency of 900 MHz was therefore used in this study. With approximately 7 billion subscriptions worldwide, mobile phones have come to represent a significant part of the modern telecommunications system. Due to the increase in their use, numerous studies have investigated the effects of the EMF on human health (ICNIRP, 2009; WHO, 2014; SCENIHR, 2015). Some of these studies have proved that EMF causes injury in numerous tissues, including the liver, kidney, testis and ovarium (Koca et al., 2013; Odacı et al., 2015; Hancı et al., 2013; Türedi et al., 2016).

Peripheral nerves (PN) are structures that provide motor, autonomic and sensory innervation for target organs in the entire body. Histologically, PN consist of two main components, nerve fibers composed of axons and the Schwann cells surrounding them, and a connective tissue sheath consisting of three layers (Carriel et al., 2014). The connective tissue sheath consists of the epineurium surrounding the PN externally, the perineurium around nerve fiber bundles and the endoneurium surrounding each nerve fiber (Klimczak et al., 2017; Jortner, 2011). PN transmit signals between the central nervous system (CNS) and target organs. Numerous studies have shown that EMF has injurious effects on the CNS, and particularly on the brain. Although numerous studies have investigated the effects of 900-MHz EMF on the CNS (Sonmez et al., 2010; Baş et al., 2013), our review of the literature revealed no previous study of its effects on the sciatic nerve, one component of the peripheral nervous system (PNS).

Mild et al. (2012) reported that mobile phones not in use emit almost no EMF, while in stand-by mode they are only active during periodic location updates every 2–5 h. Today, however, modern smart phones are used in many other ways than simply for talking and texting. For example, they are permanently on line for data exchanges. As a result, more frequent location updates are needed, and the user is therefore exposed to EMF even if the mobile phone is on stand-by mode (Urbiniello and Rössli, 2013). When in use, mobile phones are held close to the skull, in other words close to the brain. However, when not in use, or when a headphone is used, they are generally kept in back pockets or in handbags, and thus in close proximity to the sciatic nerve.

Considering that the PNS permeates the entire body and is connected to neurons in the CNS, it will not be surprising if EMF exhibiting deleterious impacts on the CNS will also affect the PNS. The purpose of this study was therefore to use histopathological and biochemical methods to investigate the effects of exposure to a 900-MHz EMF throughout adolescence on the male rat sciatic nerve.

2. Materials and methods

2.1. Study design and ethical procedures

The study began once written ethical committee authorization had been obtained from the Karadeniz Technical University (KTU) Animal Care and Ethics Committee. Twenty-four male Sprague Dawley rats, aged 21 days and weighing 34.21 ± 0.78 (mean $\pm$ SD) grams, were used in this study. These were purchased from the KTU Experimental Animal Surgical Research and Application Center (Trabzon, Turkey). Rats were kept in standard plastic laboratory cages under standard

laboratory conditions (room temperature = $22 \pm 2^\circ\text{C}$; humidity = $50\% \pm 10$) in a 12 h light/12 h dark cycle. Standard commercially available rat chow (Bayramoğlu Feed and Flour Industry Trading Corporation, Erzurum, Turkey) and tap water were provided ad libitum for all rats during the experimental procedures. All experimental and surgical procedures were designed to be compatible with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Rats were randomly divided into three equal groups ($n = 8$ for each group), control (CGr), sham (SGr) and EMF exposure (EMFGr). No procedure was performed throughout the experiment on the CGr rats. Sham group rats were placed inside EMF cage (EMFC) with an open top for 1 h every day at 10.00–11.00 a.m. throughout the experimental period (postnatal days 21 to 59), but were not exposed to EMF. EMF group rats were exposed to a continuous 900-MHz EMF for 1 h a day from 11.00 a.m. to 12.00 p.m. inside the EMFC throughout the experimental period. SGr and EMFGr rats were exposed to sham and EMF application in a separate room defined as the EMF exposure room (EMFER). Following sham and EMF exposure, both groups were returned to their cages in the room where they were housed together with the CGr rats.

2.2. EMF cage and exposure system

The EMFC was an open-top cage made from Plexiglas and specially manufactured for use in similar studies ($40.5\text{ cm} \times 31.5\text{ cm} \times 40.5\text{ cm}$ in size). In order to establish a 900-MHz EMF effect inside the EMFC, an oscillator with an output power of approximately 300 mW and adjusted to a frequency of 900-MHz (1218-BV, Lockable Oscillator, 900–2000 MHz, General Radio Company, Concord, Massachusetts, USA, Serial No. 1483) was used. A continuous power source (1267-B Regulated Power Supply, General Radio Company, Concord Massachusetts, USA, Serial No. 903) was used to attach the oscillator to the municipal electricity network. The oscillator was attached to a semi-wave dipole antenna made from a copper rod, 15 cm in length and 1 mm in diameter, with the help of a cable. The dipole antenna was attached to the middle of a wooden rod extending approximately 11 cm from the top of the EMFC and between the right and left sides.

First of all we checked that there was no other potential source of EMF when the EMF device was turned off. In order to determine the intensity of the EMF to which EMFGr rats were exposed during application, this was measured at nine different points on the internal and external surfaces of the EMFC floor. These measurements were performed using a broadband EMF measurement device (CA 43 Isotropic Electrical Field Intensity Meter, Chauvin Arnoux Group, Paris, France) operating between 100 kHz and 2.5 GHz. The measurements showed that rats were exposed to a mean 8.4 V/m electric field inside the cage (0.187 W/m^2). On the basis of these data, the specific absorption rate (SAR) was calculated at 0.0093 W/kg (RadHaz SAR Equivalency Calculator, Version 1.0.-2000 Richard Tell Associates Inc., Colville, WA).

2.3. Removal of the sciatic nerve

No rats died during the experiment or were excluded for any other reason. At the end of the experiment (postnatal day 60), all rats from all groups were sacrificed via cervical dislocation under deep anesthesia (Ketalar® 50 mg/kg, Eczacıbaşı Co., Istanbul, Turkey). A cutaneous and subcutaneous incision to the hip joint was made in order to access the sciatic nerve. The tissues surrounding the muscles were then carefully dissected, and the nerve was extracted as far as its branching point in the proximal part of the knee joint. The first 1-cm section was taken from above the branching point and half of it fixed in 10% formalin (SigmaAldrich, St. Louis, MO) for histopathological analyses. The other half was placed into Eppendorf tubes at -80°C in a deepfreeze for biochemical analysis. All analyses, histopathological evaluations and

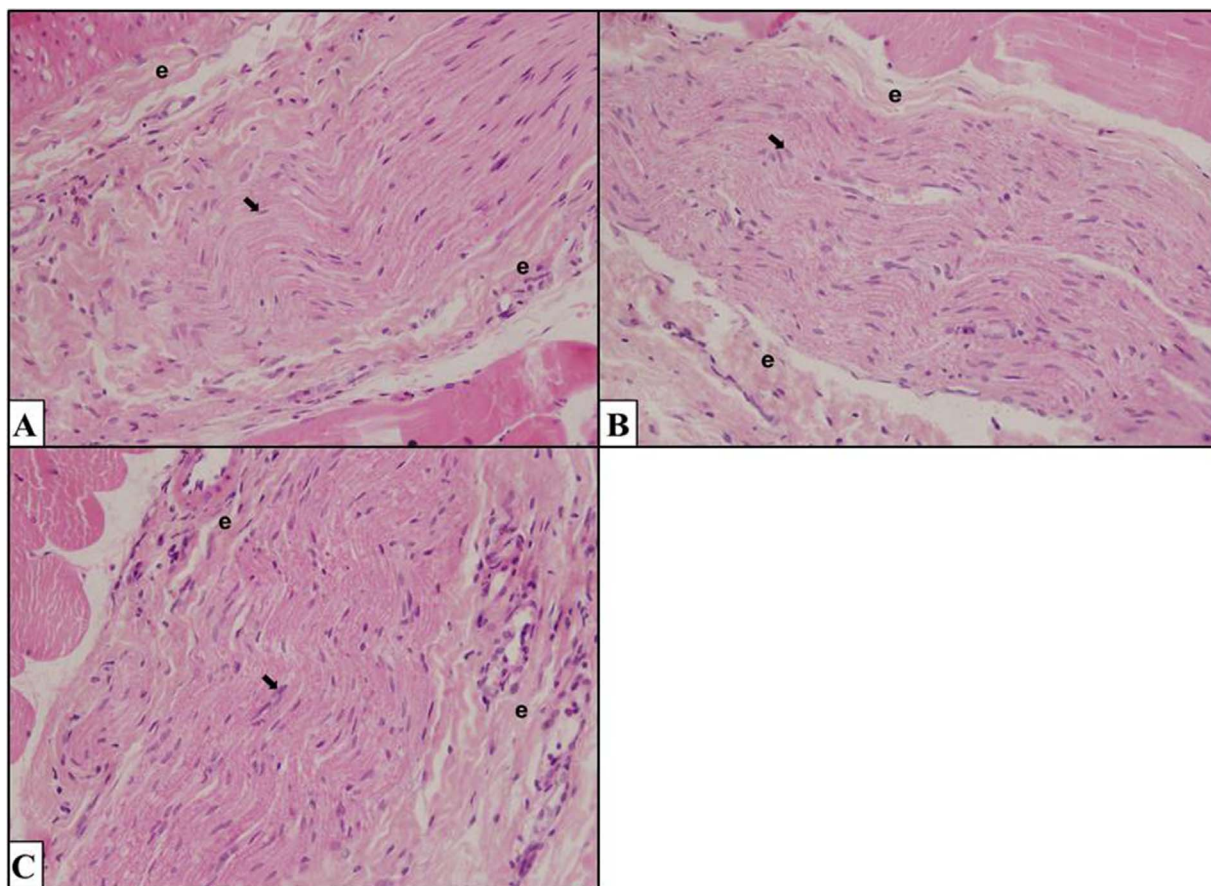


Fig. 1. Epineurium (e) surrounding the sciatic nerve of rats and nerve fibers and surrounding Schwann cell (arrow) with a normal structure were observed in sections from the control (A), sham (B) and EMF groups (C) (Hematoxylin and eosin, $\times 400$).

counting procedures were performed blind by the authors.

2.4. Histological procedures

Following 3-day fixation in 10% formalin, the sciatic nerve specimens were subjected to routine histological tissue procedures and embedded longitudinally in paraffin blocks. Tissue sections 5 μ m in thickness were taken from the paraffin blocks with the help of a microtome (Leica RM 2255, Leica Instruments, Nussloch, Germany). Sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation and with Masson's trichrome to show connective tissue sheaths and collagen in epineural tissue. For histopathological analysis, H&E and Masson's trichrome-stained sciatic nerves sections were evaluated using a research microscope (Olympus BX 51, Olympus Optical Co., Tokyo, Japan) with a DP 71 camera (Olympus, Olympus Optical Co., Tokyo, Japan) at different magnifications. Microphotographs were taken using the same research microscope.

2.5. TUNEL staining and apoptosis evaluation

Apoptosis in sciatic nerves tissues from study group rats was evaluated on the basis of DNA fragmentation in the Schwann cells and connective tissue cells without distinction using TUNEL staining, a standard terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling assay method. Next, 5- μ m sections were placed onto slides. After the deparaffinization procedures, sections were performed using an In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany, Product No = 11684817910) in accordance with the manufacturer's instructions. Apoptosis was evaluated on TUNEL-

stained slides by means of a research light microscope (Olympus BX 51 microscope; Olympus Optical Co., Tokyo, Japan) at a magnification of $\times 400$ using Analysis 5 Research software (Olympus Soft Imaging Solutions, Münster, Germany). One hundred cells in five randomly selected areas were counted on each section. Homogeneously stained TUNEL (+) cells with no areas of necrosis were defined as apoptotic if they contained a brown nucleus. At this stage, all cells with a brown nucleus, with no distinction between Schwann and connective tissue cells, were regarded as apoptotic. The apoptotic index (AI) was calculated by multiplying the total number of apoptotic cells by 100 (Odaci and Özyılmaz, 2015; Türedi et al., 2016).

2.6. Biochemical analyses

Catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) were investigated as oxidative stress inducers in tissues stored in Eppendorf tubes in a deep freeze at -80°C . CAT activity was determined following the method developed by Aebi (1984). CAT activity was defined as the quantity of enzyme needed to decompose 1 mmol of H_2O_2 per minute, at 25°C and at pH 7.8. Results were expressed as mmol/min/mg of tissue (Aebi, 1984).

Estimations of SOD enzyme activities were based on the manufacture of superoxide radicals generated by xanthine and xanthine oxidase, which reacts with nitro blue tetrazolium to form formazan. SOD activity was measured at 560 nm by the degree of inhibition of this reaction. Activity was expressed as mmol/min/mg tissue (Sun et al., 1988).

Tissue GSH levels were measured using 5, 5'-dithiobis (2-nitrobenzoic acid) following the modifying method described by Sedlak and Lindsay (1968). The absorbance was measured at 412 nm, and

tissue GSH levels were expressed as nmol/mg tissue.

Tissue lipid peroxidation (LPO) levels were determined based on the method described by [Ohkawa et al. \(1979\)](#). Tissue LPO levels were determined by estimating MDA levels using the thiobarbituric acid test. The tissues were scraped, weighed and homogenized in 10 ml of 100 g/l KCl. The absorbance of supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. The recovery rate was over 99%. The results were expressed as nmol/g tissue ([Ohkawa et al., 1979](#)).

2.7. Statistical analysis

Statistical analysis of the study data was performed on Statistical Package for the Social Sciences (IBM SPSS Statistics for Windows 23.0, Chicago, IL, USA) software. Kruskal Wallis analysis of variance was used to compare differences between group parameters. Dual comparisons between groups exhibiting significant values were evaluated using the Mann–Whitney *U* test with Bonferroni correction. All results are presented as means $\pm$ standard deviation. Significance was set at $p \leq 0.01$ for all tests.

3. Results

3.1. Histopathological analysis results

Preparates stained with H&E from CGr ([Fig. 1A](#)), SGr ([Fig. 1B](#)) and EMFGr ([Fig. 1C](#)) were analyzed under a light microscope at different magnifications. This showed that the sciatic nerves had a normal structure, and no pathology was observed. Evaluation of sections stained with Masson's trichrome from CGr ([Fig. 2A](#)) and SGr ([Fig. 2B](#))

also revealed that the sciatic nerves had a normal structure. However, the epineurial layer of the sciatic nerve was markedly thicker in sections stained with Masson's trichrome from EMFGr ([Fig. 2C](#)).

3.2. TUNEL assay and apoptosis evaluation results

When EMFGr sections stained using the TUNEL method were compared with those from CGr and SGr, while a small number of apoptotic cells was seen in CGr ([Fig. 3A](#)) and SGr sections ([Fig. 3B](#)), abundant and randomly distributed apoptotic cells were observed in the EMFGr sections ([Fig. 3C](#)). AI analysis revealed a significant increase in the number of TUNEL (+) cells when EMFGr was compared with CGr and SGr ($p \leq 0.01$ and $p \leq 0.01$, respectively) ([Table 1](#)).

3.3. Biochemical analysis results

Statistical analysis revealed no statistically significant difference in any biochemical analysis results from rats from CGr and those from SGr ($p > 0.01$), except for SGr SOD levels, which were higher than those in CGr ($p \leq 0.01$ [Table 1](#)). Statistical comparison of biochemical analysis results from EMFGr with those from CGr and SGr rats revealed a significant increase in MDA, SOD and CAT values ($p \leq 0.01$ [Table 1](#)).

4. Discussion

EMF emitted by mobile phones and other devices in modern life is a diffuse environmental agent, and the EMF produced by these can cause peripheral neurophysiological effects in some individuals ([Westerman and Hocking, 2004](#)). Experimental studies investigating the effects of EMF on biological systems have reported damage in several organs,

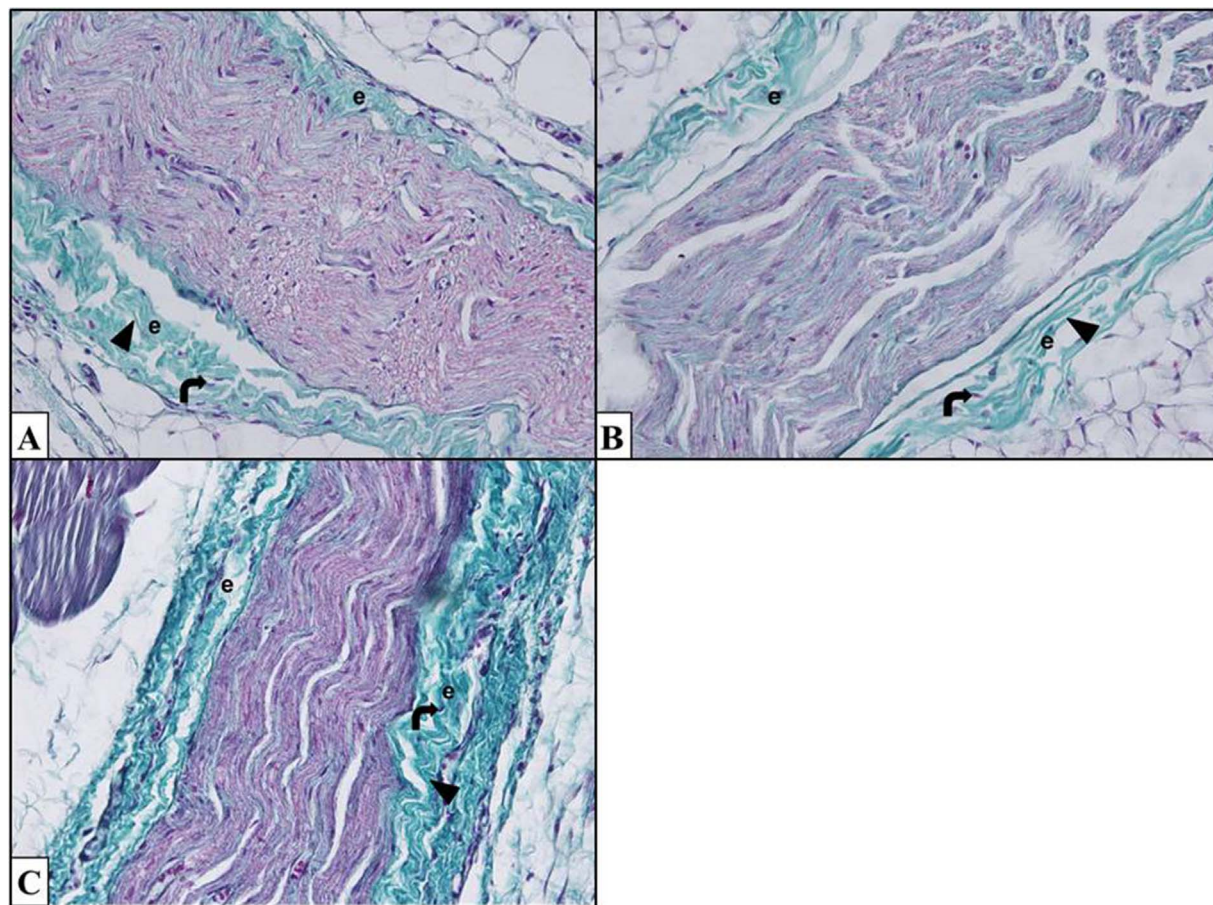


Fig. 2. Thickening in the epineurium (e), collagen fibers (arrowhead) and fibrocytes (curved arrow) were observed in sections from the EMF group (C) compared to sections from control (A) and sham groups (B) (Masson's trichrome, $\times 400$).

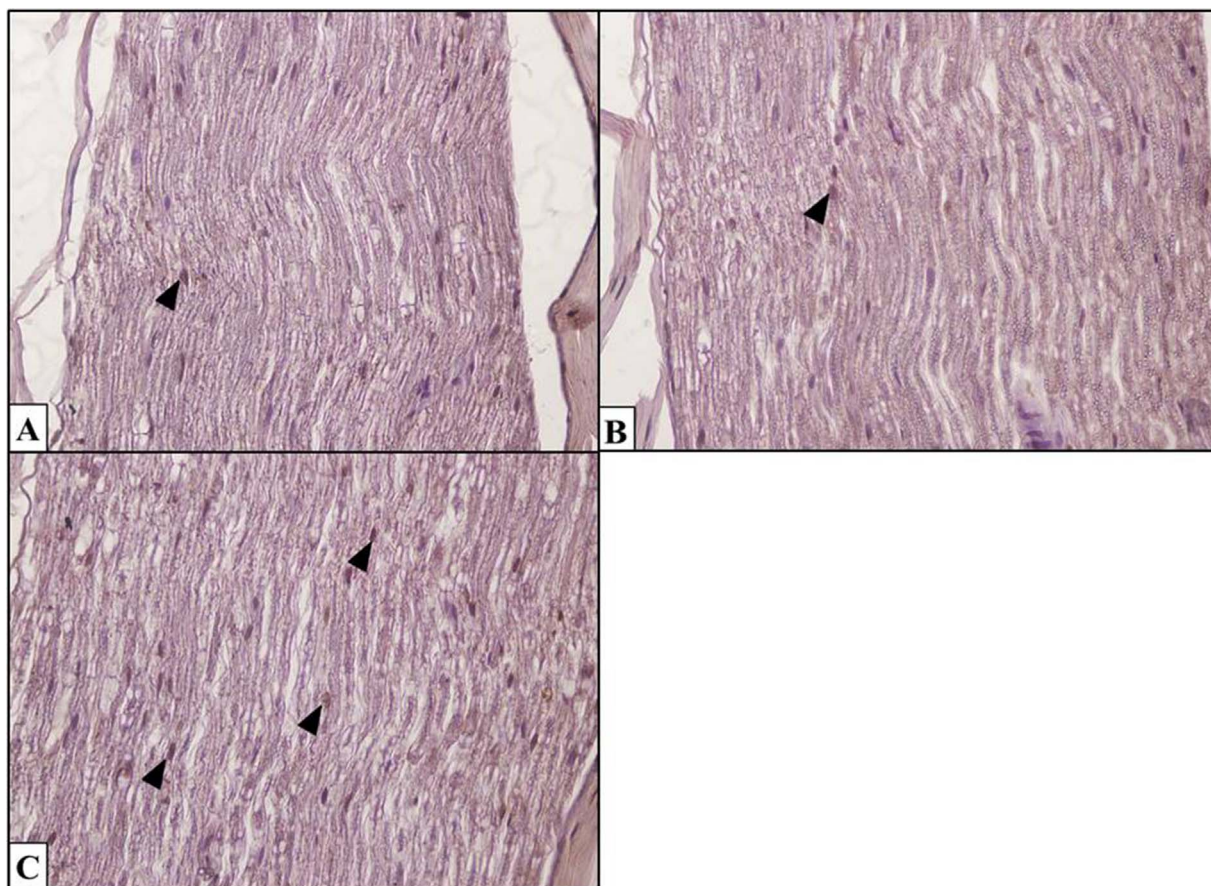


Fig. 3. A marked increase can be seen in apoptotic cells (arrowhead) with a brown nucleus in sections from the EMF group (C) compared to sections from control (A) and sham groups (B) (TUNEL, $\times 400$).

Table 1

Statistical analyses of apoptotic index and biochemical analysis results from the sciatic nerve tissues from the study group rats.

Biochemical parameters	Control group (n = 8)	Sham group (n = 8)	EMF Group (n = 8)
MDA(nmol/mg tissue)	13.68 $\pm$ 0.90	14.66 $\pm$ 0.37	20.31 ^a $\pm$ 1.85
CAT(mmol/min/mg tissue)	45.96 $\pm$ 7.89	57.81 $\pm$ 7.52	116.18 ^a $\pm$ 21.87
SOD(nmol/min/mg tissue)	0.04 $\pm$ 0.00	0.09 ^b $\pm$ 0.06	0.21 ^a $\pm$ 0.03
GSH(nmol/mg/tissue)	0.55 $\pm$ 0.01	0.58 $\pm$ 0.02	0.57 $\pm$ 0.04
Apoptotic index (%)	11.5 $\pm$ 4.3	13.1 $\pm$ 3.5	28.8 ^a $\pm$ 4.0

The data represent means $\pm$ SD.

^a EMF group values increased significantly compared to the Control group and Sham group ($p \leq 0.01$, $p \leq 0.01$ respectively).

^b Sham group values increased compared to the Control group ($p \leq 0.01$).

including the brain, liver, kidney and heart (Baş et al., 2013; Topal et al., 2015; Odacı et al., 2015; Türedi et al., 2015). The effects of EMF on the brain in particular have attracted considerable scientific interest (Çam et al., 2014). However, no previous studies have investigated the effects of EMF on the sciatic nerve of adolescent rats at the morphological level. Any change that may occur in the morphology of the PNS may impair communication from the center to the periphery, or from the periphery to the center.

Although the pathophysiology of the adverse effects caused by EMF is uncertain; oxidative stress, resulting from an increase in ROS and impairment of cellular antioxidant defense mechanisms, may explain

the pathologies it causes in tissues (Terzi et al., 2016). MDA, an end product of ROS attack on membrane lipids, is an important marker of oxidative stress in tissues (Valko et al., 2007). One study reported that exposure to a 900-MHz EMF increased MDA levels in the spinal cord (Kerimoğlu et al., 2016b), and other studies have reported that it increased MDA levels in the brain (Bilgici et al., 2013; Meral et al., 2007). In our study, and in agreement with the literature, MDA levels increased in sciatic nerve tissue from EMFGr.

Antioxidant defense mechanisms protecting the body against the deleterious effects of ROS include enzymatic molecules such as SOD and CAT or non-enzymatic molecules such as GSH (Valko et al., 2007). Meral et al. (2007) reported that the application of a 900-MHz EMF caused a decrease in GSH and CAT levels in the brain, while Kerimoğlu et al. (2016b) reported a decrease in SOD and GSH levels in the spinal cord. Our study findings were not compatible with those studies. However, similarly to our study, Türedi et al. (2015) observed an increase in SOD and CAT levels in the hearts of rat pups of pregnant rats exposed to a 900-MHz EMF. Another study reported high serum SOD in an EMF group, as in our study, but in contrast to the present study, brain SOD levels were no different to those in the other groups (Irmak et al., 2002). The high SOD and CAT values in EMFGr in our study may be interpreted as indicating a defense response against increasing ROS attack. In contrast to the high SOD and CAT values in EMFGr, GSH values in the same group did not increase significantly in that group compared to the control group. This may be attributed to a higher level of GSH being used to eliminate increased superoxide radicals (Yurekli et al., 2006). Differences in the literature in terms of changes in amounts of antioxidant molecules caused by exposure to EMF may have derived from differences in tissues, lengths of application and application methods.

Numerous studies have shown that cage cleaning and transportation to another cage or room can cause a stress response in experimental animals (Castelhano-Carlos and Baumans, 2009). Abnormal physiological or emotional states causing stress can lead to oxidative stress by impairing cellular homeostasis through various mechanisms (Samarghandian et al., 2017). Since SOD levels were higher in SGr than in CGr, we speculated that oxidative stress for which the organism was seeking to compensate might have developed in SGr. However, since MDA levels and AI were similar to the values observed in CGr, the oxidative stress may have been compensated by the action of antioxidant mechanisms.

Oxidative stress is involved in the pathophysiology of numerous diseases, such as cancer, type II diabetes, arteriosclerosis, chronic inflammatory processes, and various neurodegenerative diseases (Valiko et al., 2007). ROS responsible for oxidative stress not only affect membrane lipids, but can also trigger apoptosis by causing oxidation of macromolecules such as proteins, DNA damage and mitochondrial irregularities (Ott et al., 2007; Hou et al., 2015). Indeed, different studies have reported that exposure to EMF increases apoptosis in such tissues as the heart, testis and cochlea (Türedi et al., 2015; Odacı et al., 2016; Çeliker et al., 2017). Liu et al. (2012) observed that in vitro exposure to 1950-MHz EMF caused mitochondrial damage in astrocytes, glial cells of the CNS, and induced apoptosis via the caspase 3 pathway. The increased apoptosis in sciatic nerve cells in our study, in agreement with the previous literature, was therefore an expected finding. We think that the increased apoptosis was induced by oxidative stress.

We observed a thickening in epineurial layers of sciatic nerves from EMFGr at the light microscopic level. The epineurium, a connective tissue sheath around the entire sciatic nerve, is rich in type 1 and 3 collagen fibers (Klimczak et al., 2017). Tzaphlidou and Fotiou (2004) reported that exposure to 910-MHz EMF for 2 h a day over 30 days can impair the axial periodicity of collagen fibers in the dura mater and arachnoid mater layers around the CNS and may be responsible for structural abnormalities in collagen. Other studies have reported that exposure to 50-Hz EMF causes an increase in connective tissue between follicles in the rat thyroid (Mohamed and Elnegris, 2015; Rajkovic et al., 2006). We therefore hypothesized that the thickening observed in the epineurium in EMFGr in our study may have occurred in association with an increase in secondary connective tissue and/or probable structural changes in collagen fiber secondary to the effect of EMF.

In conclusion, exposure to a 900-MHz EMF throughout adolescence may result in oxidative stress and apoptosis in the sciatic nerves of rats at the beginning of adulthood. However, considering the nervous system as a whole, it is not possible to answer the question of whether this effect is a secondary effect of EMF on the CNS or else a primary effect of EMF on the PNS on the basis of our study findings. Other studies may be planned in order to answer this question. Nonetheless, considering that a pathological effect beginning in adolescence may increase cumulatively with age, we anticipate that exposure to EMF in adolescent rats may cause sciatic nerve damage that may be encountered in adulthood.

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